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Genetic characterization and molecular epidemiology of foot-and-mouth disease viruses isolated from Afghanistan in 2003–2005

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Abstract Foot-and-mouth disease virus (FMDV) isolates collected from various geographic locations in Afghanistan between 2003 and 2005 were genetically characterized, and their phylogeny was reconstructed utilizing nucleotide sequences of the complete VP1 coding region. Three serotypes of FMDV (types A, O, and Asia 1) were identified as causing clinical disease in Afghanistan during this period. Phylogenetic analysis revealed that the type A viruses were most closely related to isolates collected in Iran during 2002–2004. This is the first published report of serotype A in Afghanistan since 1975, therefore indicating the need for inclusion of serotype A in vaccine formulations that will be used to control disease outbreaks in this country. Serotype O virus isolates were closely related to PanAsia strains, including those that originated from Bhutan and Nepal during 2003–2004. The Asia 1 viruses, collected along the northern and eastern borders of Afghanistan, were most closely related to FMDV isolates collected in Pakistan during 2003 and 2004. Data obtained from this study provide valuable information on the FMDV serotypes circulating in Afghanistan and their genetic relationship with strains causing FMD in neighboring countries.

Keywords FMDV · VP1 · Phylogenetic analysis · Nucleotide · Amino acid · Afghanistan

Introduction

Foot-and-mouth disease (FMD) is an extremely contagious and economically devastating disease of cloven-hoofed animals. FMD virus (FMDV) is a single-stranded, non-

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segmented, positive sense RNA virus of approximately 8.2 kb that belongs to the Picornaviridae family, genus *Aphthovirus* [1]. There are seven FMDV serotypes (types A, O, C, Asia 1, and South African Territories (SAT) types 1–3) and many intratypic variants [2–4]. Currently, FMD is not present in North or Central America, Australia, the European Union or New Zealand. However, it is endemic in parts of Asia, Africa, the Middle East, and South America, and is the cause of outbreaks similar to those observed in the United Kingdom in 2001 [5].

FMD is endemic in Afghanistan and its neighboring countries in the Middle East-South Asia (ME-SA) region [5–7]. Three of the seven FMD serotypes (types O, Asia 1, and A) are prevalent throughout this region [4]. India, Pakistan, and Iran have reported the presence of serotypes O, Asia 1, and A (2001–2006), while Afghanistan has reported the presence of serotypes O and Asia 1 during a similar period (2001–2003) [5]. Serotype O is the most prevalent and widely distributed serotype of FMD virus on a global scale [8]. Asia 1 is primarily restricted to Asia with recent incursions into the ME region and Greece [9, 10]. Serotype A is the cause of disease in the ME-SA region and is the most antigenically diverse of the seven serotypes [4, 11, 12].

The epidemiology of FMD in the ME-SA region is complex. Efforts to control virus movement in this region are complicated by the fact that border control is limited or nonexistent, there is a large population of nomadic herds, animal movement is unrestricted, there is no established system for reporting or responding to disease outbreaks, and communication between provincial veterinarians and the central government is difficult.

Iranian [13] and Indian [6, 14–16] FMDV isolates (types A, O, and Asia 1) have been well characterized both antigenically and phylogenetically. In addition, the World Reference Laboratory (WRL) for FMD located in Pirbright, UK, maintains a database of VP1 capsid sequences from FMDV isolates collected around the world [4, 8, 10, 17].

Since 2001, there have been few reports on the status of FMD in Afghanistan. Epidemiological data and genetic characterization of circulating field viruses as well as their relationship to vaccine strains being employed in this country have not been reported. In order to control FMD in Afghanistan, it is necessary to understand the complex epidemiological relationships that are occurring secondary to unrestricted animal movement. Implementation of an effective vaccination program will require characterization of currently circulating field isolates and continued monitoring to ensure that vaccine strains are protective against field viruses.

During the period from 2003 to 2005, through a collaboration between the government of Afghanistan, the United States Department of Defense (DOD) and the United States Department of Agriculture (USDA), field isolates were collected and shipped to the Plum Island Animal Disease Center (PIADC) in Orient Point, New York for antigenic and genetic characterization. DOD and Afghan veterinarians collected samples from animals with clinical signs of FMD, clinically normal animals on the same premises and animals from premises with no known FMD cases. Here we report the serotype of FMD viruses circulating in Afghanistan during 2003–2005, genetically characterize these isolates, and employ phylogenetic analysis to determine their relationship to FMD strains circulating in other countries.

Materials and methods

Sample collection

Animals from 11 provinces within Afghanistan (Kabul, Zabul, Nangarhar, Parwan, Hirat, Ghazni, Konduz, Balkh, Kapisa, Saripol, and Paktika) were sampled as part of veterinary visits to villages and farms. Specimens were collected from ruminants showing clinical signs (fever, depression, hypersalivation, lameness, vesicles, loss of appetite and weight), those who had been potentially exposed, and those with no known exposure to FMDV. A total of 116 oral swabs (79 bovine, 18 ovine, 15 caprine, and four not specified) and 20 epithelial samples (14 bovine, four ovine, and two caprine) were collected between 2003 and 2005. The four ovine and 13 of the 14 bovine epithelium samples also had an oral swab collected from the same animal, while the two caprine epithelium samples did not have a corresponding oral swab. Sampling was based on accessibility by vehicle and was not designed to be representative of the regions or the entire country. Oral swabs were collected with a sterile Dacron swab and placed in 1.5 ml Dulbecco's Modified Eagle Medium (DMEM) containing 1× antibiotic/antimycotic. Epithelial samples were collected in a 50% glycerol:50% 0.04 M phosphate buffered saline (PBS) solution. Following collection, samples were immediately placed in a cooler containing ice for transport to Bagram Air Field, Afghanistan. Once the samples arrived at Bagram Air Field, they were stored at 4°C until shipment to the Foreign Animal Disease Diagnostic Laboratory (FADDL), PIADC, Orient Point, NY. Upon arrival at FADDL, samples were inventoried and placed at −70°C until further use.

Sample processing

Oral swabs

For RNA isolation, 140 µl of each sample was placed in 560 µl of RLT lysis solution (RNeasy kit, Qiagen). For

virus isolation (VI), 500 µl aliquots were clarified by centrifugation through a 0.22 µm Spin-X® cellulose acetate centrifuge tube filter (Corning). The remaining sample was frozen at −70°C for future use.

Epithelium

Epithelial tissues were washed three times in 1× PBS, pH 7.4. For RNA extraction, 30 mg of each epithelial sample was placed in 600 µl of RLT buffer and subsequently macerated with a sterile 1.5 ml pestle. The homogenate was clarified through a Qiashredder (Qiagen) by centrifugation in a table-top centrifuge at maximum speed (~16,000*g*). For VI, 10% homogenates of each epithelial tissue were prepared and filtered through a 0.22 µm Spin-X® cellulose acetate centrifuge tube filter (Corning). The remainder of the sample was frozen at −70°C for future use.

Virus isolation

Clarified oral swab or epithelium samples were tested on lamb kidney (LK), and/or Instituto Biologico Rim Suino 2 (IBRS-2, swine kidney) cells. Samples (500 µl) were inoculated on approximately 90% confluent T-25 flasks and incubated at 37°C, 5% CO_2 in a humidified incubator. Cells were monitored for cytopathic effect (CPE) daily, and frozen when CPE was exhibited or at 72 h post-infection (hpi). A second pass was performed on those samples not presenting CPE following the same procedure as the first pass. Samples not exhibiting CPE by 72 hpi on the second pass were considered VI negative.

RNA isolation

RNA was extracted from epithelium and oral swabs using the RNeasy Mini Kit (Qiagen). Briefly, after sample processing, 700 µl of 70% ethanol was added to the oral swab or tissue lysate, gently mixed, and transferred to an RNeasy spin column and centrifuged for 30 s at 16,000*g*. This process was repeated for the remaining lysed sample/70% ethanol mixture. This was followed by addition of RW1 wash buffer, two washes with RPE buffer, a dry spin, and elution with 40 µl RNase-free water.

Detection of FMD viral RNA by rRT-PCR

Real-time RT-PCR (rRT-PCR) reactions were performed using a "dried-down", single-step, one-tube, rRT-PCR assay designed and manufactured by Tetracore, Inc. (Gaithersburg, MD). Final concentrations of reagents have been previously published [18]. Thermal cycling conditions for the Cepheid SmartCycler™ II were 60°C for 10 min, followed by 45 cycles at 95°C for 2 s, and 60°C for 30 s. Total assay time on the Cepheid SmartCycler™ II was approximately 1 h. Cycle threshold (Ct) results of the FMDV rRT-PCR were called positive between cycles 0.00–40.00, and inconclusive between cycles 40.00–45.00 (based on unpublished validation studies).

RT-PCR and sequencing

Samples testing positive by the FMDV rRT-PCR assay were further analyzed by nucleotide sequencing of the VP1 capsid region of the genome. RNA was reverse transcribed (RT) with random primers using the StrataScript™ First Strand cDNA Synthesis Kit (Stratagene®). The VP1 coding region was amplified using the Advantage™ cDNA PCR Kit (BD Biosciences) according to manufacturer's protocol. Previously published [19] and newly designed primers were optimized for PCR amplification (Table 1). PCR products were analyzed by agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen) per manufacturer's protocol. Purified products were sequenced on a 3730*xl* DNA Analyzer (Applied Biosystems) by dideoxy-sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All nucleotide sequences were obtained from clinical sample RNA by direct sequencing of PCR products.

Phylogenetic analysis

Complete VP1 nucleotide sequences of each isolate used in this study have been submitted to GenBank, and the accession numbers are listed in Table 2. Isolate names have been abbreviated using the following format: serotype/city or region (if available)/three-letter country code/isolate number/year. The three-letter country codes are designated as outlined by the World Reference Laboratory for FMD (WRLFMD). Alignments and contigs of nucleotide sequence data were assembled using Sequencher™ 4.7 software (Gene Codes Corporation). Consensus sequence for each isolate was derived from at least three independent forward and reverse sequences. Complete VP1 sequences from other sources were downloaded from the Entrez Nucleotide database, National Center for Biotechnology Information and from the WRL, Pirbright, UK. Alignments were performed using Clustal X. Phylogenetic relationships were reconstructed utilizing maximum likelihood (ML) analysis (PAUP* beta10 version, Sinauer Associates,

Table 1 Primer sequences and cycling profiles used to amplify the VP1 region

Primer	Sequence 5′ → 3′	Sense	Genome location	Cycling conditions	Serotype amplified	Reference
HF-28	GGCGCAGTACTACACACA	+	VP3	1	O	[20]
HR-34	CGTCGGAGAAGAAGAAGGG	–	2B	1	O	[20]
O1F	CCAACCCAACNGCTTACCACA	+	VP1	2	O	Steve Pauszek, personal communication
Rev1	ACAGCGGCCATGCAYGACA	–	2B	2	O	Steve Pauszek, personal communication
HF-29	TGAGTGGGACACTGGTCT	+	VP3	1	Asia	[20]
HR-34	CGTCGGAGAAGAAGAAGGG	–	2B	1	Asia	[20]
A-1C562	TACCAAATTACACACGGGAA	+	VP3	3	A	[19]
NK61	GACATGTCCTCCTGCATCTG	–	2B	3	A	[19]

Cycling conditions: 1 → 95°C 5 min, 35 cycles of (95°C 15 s, 50–55°C for 15 s, 68°C for 2 min), 68°C 10 min; 2 → 94°C 5 min, 35 cycles of (94°C 30 s, 50–55°C for 20 s, 68°C for 2 min), 68°C 10 min; 3 → 94°C 5 min, 35 cycles of (94°C 1 min, 55°C for 1 min, 72°C for 1.5 min), 72°C 10 min

Inc.). Similar topologies were observed when neighbor joining (NJ) or maximum parsimony (MP) analyses were performed. Bootstrap analysis was performed on MP generated trees (2,000 replicates for 95% reproducibility) as computing power was not available for bootstrap analysis on ML generated trees. In order to provide perspective to the phylogenetic trees, O_1/Manisa/TUR/69 was used as the outgroup for the serotype A and Asia 1 phylogenetic trees and A/IND/455/98 was used as the outgroup for the serotype O phylogenetic tree.

Results

FMDV status of the isolates was based on a combination of rRT-PCR, VI, and nucleotide sequencing results. All samples were tested by rRT-PCR and VI, while nucleotide sequencing was attempted on those samples testing positive by rRT-PCR (all VI positive samples were initially rRT-PCR positive). The 15 caprine, 18 ovine, and four unspecified species oral swab samples tested negative for the presence of FMDV by rRT-PCR and VI. Bovine oral swabs samples tested by rRT-PCR resulted in 61 negative (all VI negative), 16 positive (three VI positive), and two inconclusive (both VI negative) samples. One inconclusive sample was negative by VI and no sequence could be obtained, therefore this sample was considered negative. The other inconclusive sample was considered positive even though it was negative by VI, because a complete VP1 nucleotide sequence was obtained. The four ovine epithelium samples were negative by rRT-PCR and VI, while one of the two-caprine samples was positive (both VI negative). Ten of the 14 bovine epithelium samples were positive by rRT-PCR (three VI positive). When there was an oral swab and epithelium sample from the same animal, rRT-PCR results showed six of the 13 bovine samples to be positive in both sample matrices, three both negative, two positive epithelium/negative oral swab, one negative epithelium/positive oral swab, and one positive epithelium/inconclusive oral swab. Ovine oral swab and epithelium samples collected from the same animal (four instances) were all negative.

On the basis of rRT-PCR positive or inconclusive results, nucleotide sequence of the complete VP1 region was obtained for 24 isolates as follows: 14 of the 16 positive bovine oral swabs, 1 of the 2 inconclusive bovine oral swabs, eight of the 10 positive bovine epithelium samples, and the one positive caprine epithelium sample. Six of the sequences were obtained from an oral swab and epithelium sample from the same animal, leaving 18 complete VP1 sequences from individual animals. This study reports the phylogenetic relationships of those 18 FMDV isolates (Table 3). While nucleotide sequencing of these isolates technically groups them into genotypes (due to sample volume limitations only a few isolates were able to be serologically tested), the isolate designations are termed serotypes to maintain consistency with comparable literature and since a strong correlation exists between serotype and phylogenetic designation. Serotypes A, O, and Asia 1 were found to be circulating during this time period and have been previously reported [5, 22]. Phylogenetic analyses further defined genetic relationships of these viruses with available complete VP1 nucleotide sequences of viruses circulating in the ME-SA region.

A total of five serotype A viruses were identified and characterized. Two were located in Nangarhar located on the eastern side of the country while the other three serotype A viruses were collected on the same farm on the western side of the country in Hirat (Fig. 1). Two of the three isolates collected in Hirat were identical in VP1

Table 2 List of isolates used in phylogenetic analysis

Isolate	Host species	GenBank Accession No.	Isolate	Host species	GenBank Accession No.
A/AFG/130/2004	Bovine	EF457980	Asia1/PAK/20/2003*	NK	DQ121126
A/AFG/131/2004	Bovine	EF457981	Asia1/PAK/30/2002*	Buffalo	DQ121124
A/AFG/160/2005	Bovine	EF457982	Asia1/PAK/33/2002*	Buffalo	DQ121125
A/AFG/183/2005	Bovine	EF457983	Asia1/PAK/69/2003*	NK	DQ121127
A_{24}/ARG/65	NK	AY593767	Asia1/TAI/1/98*	Buffalo	DQ121129
A_{76}/ARG/76	NK	AJ409219	Asia1/USSR/48[a]	NK	U87835
A/BHU/41/2002*	Bovine	EU414525	O/AFG/16/2003*	Ovine	DQ165035
A_{24}/Cruzeiro/BRA/55	Bovine	AJ251476	O/AFG/50/2003*	Bovine	DQ165036
A_{27}/COL/67	NK	AY593771	O/AFG/120/2004	Bovine	EF457984
A/GAM/52/98*	NK	AF390862	O/AFG/201/2004	Caprine	EF457985
A_{10}/HOL/42	Bovine	M20715	O/AFG/210/2004	Bovine	EF457986
A_{22}/IND/17/77	Bovine	AF204108	O/ALG/1/99*	Bovine	AJ303481
A/IND/21/90	Bovine	AF390620	O_1/ARG/39	NK	AY593825
A/IND/455/98	Bovine	AF390650	O/BAR/8/98*	Bovine	AJ318825
A/IRN/1/96*	Bovine	EF208771	O/BHU/24/2003*	Bovine	DQ165040
A/IRN/2/87*	Bovine	EF208770	O/BHU/33/2004*	Bovine	DQ165046
A/IRN/2/2002*	Bovine	EU414527	O/BHU/41/2003*	Bovine	DQ165041
A/IRN/7/2003*	Bovine	EU414528	O_1/Campos/BRA/58	NK	AJ320488
A/IRN/7/2004*	Bovine	EU414530	O/BUR/6/89*	Swine	AJ294905
A/IRN/10/2003*	Bovine	EU414529	O/CAM/3/98*	Bovine	AJ294910
A/IRN/22/99*	Bovine	EF208772	O/CIV/8/99*	Bovine	AJ303485
A/IRN/34/2001*	Bovine	EU414526	O/GHA/5/93*	Bovine	AJ303488
A/IRQ/100/2002*	Bovine	EU414531	O/1696/GRG/97	NK	AJ318834
A_{21}/Lumbwa/KEN/64	NK	AY593761	O/HKN/2002	Porcine	AY317098
A/K37/84 (Kenya)	NK	EU414532	O/HKN/14/82*	Porcine	AJ294917
A/MAY/2/2002*	Bovine	EU414533	O/HKN/17/82*	Bovine	AJ294918
A/PAK/9/2003*	Bovine	EU414535	O/HKN/21/70*	Porcine	AJ294911
A/PAK/28/2002*	Bovine	EU414534	O/IRN/6/2004*	Ovine	DQ165053
A/SAU/23/86*	Bovine	EU414536	O/IRN/8/2004*	Bovine	DQ165054
A_5/SPA/86	NK	M72587	O/IRN/9/99*	NK	AJ318838
A/TAI/118/87	NK	EF208777	O/IRN/15/2004*	Bovine	DQ165055
Asia1/AFG/4/2001*	Bovine	DQ121110	O/IRN/16/2003*	Bovine	DQ165052
Asia1/AFG/22/2003	Bovine	EF457987	O/IRQ/30/2000*	Bovine	DQ165057
Asia1/AFG/24/2003	Bovine	EF457988	O_{11}/ISA/1/62*	Bovine	AJ303500
Asia1/AFG/26/2003	Bovine	EF457989	O/ISA/1/74*	Bovine	AJ303501
As/AFG/33/2003	Bovine	EF457990	O/ISA/8/83*	Bovine	AJ303503
Asia1/AFG/40/2003	Bovine	EF457991	O/ISA/9/74*	Bovine	AJ303502
Asia1/AFG/44/2003	Bovine	EF457992	O_2/Brescia/ITL/47	NK	AY593826
Asia1/AFG/116/2004	Bovine	EF457993	O/JAV/5/72*	NK	AJ303509
Asia1/AFG/138/2004	Bovine	EF457994	O/JPN/2000	Bovine	AB079061
Asia1/BAN/4/96	NK	Tubingen◆	O/KEN/2/95*	NK	AJ303514
Asia1/BAN/5/87	NK	Tubingen◆	O/KEN/83/79	Bovine	AJ303511
Asia1/BHU/27/2002*	NK	DQ121111	O/LAO/2/2000*	NK	AJ318844
Asia1/YNBS/CHA/58	Bovine	AY390432	O/Madras/IND/75	NK	AY145897
Asia1/GRE/2/2000*	NK	DQ121113	O/MAY/2/2000*	Bovine	AJ318846
Asia1/IND/1/95	Bovine	AF390683	O/MAY/6/2003*	NK	DQ165058
Asia1/IND/2/90	Bovine	AF392912	O/MOG/2000	Bovine	AJ318847
Asia1/IND/13/91	Ovine	AF390677	O/MYA/1/98*	Bovine	AJ303521

Table 2 continued

Isolate	Host species	GenBank Accession No.	Isolate	Host species	GenBank Accession No.
Asia1/IND/22/88	Bovine	AF390685	O/NEP/4/2003*	Bovine	DQ165059
Asia1/IND/63/72	NK	AF292106	O/NEP/5/2003*	Bovine	DQ165060
Asia1//IND/75/86	Bovine	AF390702	O/PAK/12/2003*	NK	DQ165066
Asia1//IND/390/97	Bovine	AF392940	O/PAK/18/2002*	Bovine	DQ165064
Asia1//IRN/4/2001*	Bovine	DQ121118	O_7/POL/59	NK	AY593830
Asia1/IRN/10/2004*	Bovine	DQ1211	O/SAR//2000	Bovine	AJ539140
Asia1/IRN/25/2004*	Bovine	DQ121120	O/SAU/38/98*	Bovine	AJ318852
Asia1/IRN/31/2004*	NK	DQ121121	O/SKR/2000	Bovine	AJ539139
Asia1/IRN/58/99*	NK	DQ121122	O/TAN/7/98*	Bovine	AJ296320
Asia1/Shamir/ISR/89	NK	Tubingen◆	O/TAW/2/99*	Bovine	AJ294927
Asia1/Kfaar/Kela/LEB/83	Bovine	AJ294931	O_1/Manisa/TUR/69	Bovine	AJ251477
Asia1/MYA/2/2001*	Bovine	DQ121123	O/UGA/5/96*	Bovine	AJ296327
Asia1/PAK/1/54*	NK	AJ251478	O/UKG/11/2001*	Porcine	AJ311723
Asia1/PAK/54	NK	AY593795	O/VIT/3/97*	Porcine	AJ294930
Asia1/PAK/1/2004*	NK	DQ121128			

NK = not known

Tubingen◆ = Sequences were provided to Nick Knowles by Otfried Marquardt [21]

* World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) sequence

[a] Year of collection not known

Table 3 Epidemiological information and laboratory results of Afghanistan isolates with complete VP1 nucleotide sequence

Animal No.	Province	Farm	Date collected	Species	Sample type	Ct value	Virus isolation	Sequence serotype	Sequence variant
22	Nangarhar	5	Dec03	Bovine	Oral Swab	29.01	–	Asia 1	AsA
24	Nangarhar	5	Dec03	Bovine	Oral Swab	30.91	–	Asia 1	AsB
26	Balkh	6	2003	Bovine	Oral Swab	26.55	–	Asia 1	AsE
26	Balkh	6	2003	Bovine	Epithelium	21.76	–	Asia 1	AsE
27	Balkh	6	2003	Bovine	Oral Swab	24.27	+	Asia 1	AsE
33	Nangarhar	17	17Dec03	Bovine	Oral Swab	28.66	–	Asia 1	AsF
33	Nangarhar	17	17Dec03	Bovine	Epithelium	18.31	–	Asia 1	AsF
40	Kapisa	21	29Dec03	Bovine	Oral Swab	29.00	–	Asia 1	AsH
40	Kapisa	21	29Dec03	Bovine	Epithelium	15.64	–	Asia 1	AsH
44	Kapisa	22	29Dec03	Bovine	Oral Swab	29.28	+	Asia 1	AsG
44	Kapisa	22	29Dec03	Bovine	Epithelium	16.94	–	Asia 1	AsG
116	Balkh	109	17Mar04	Bovine	Oral Swab	23.55	–	Asia 1	AsD
116	Balkh	109	17Mar04	Bovine	Epithelium	16.85	–	Asia 1	AsD
120	Ghazni	111	17Feb04	Bovine	Oral Swab	27.52	–	O	Oa
130	Hirat	127	9Feb04	Bovine	Oral Swab	28.49	+	A	Aa
131	Hirat	127	9Feb04	Bovine	Oral Swab	30.95	–	A	Ab
132	Hirat	127	9Feb04	Bovine	Oral Swab	33.76	+	A	Ab
138	Ghazni	129	13Feb04	Bovine	Oral Swab	33.11	–	Asia 1	AsC
160	Nangarhar	306	5Jun05	Bovine	Epithelium	15.73	+	A	Ac
183	Nangarhar	309	6Jun05	Bovine	Oral Swab	19.55	–	A	Ad
201	Zabul	214	17Feb04	Caprine	Epithelium	24.63	–	O	Ob
209	Ghazni	239	12Aug04	Bovine	Epithelium	24.27	–	O	Oc
210	Ghazni	239	12Aug04	Bovine	Oral Swab	42.97	–	O	Oc
210	Ghazni	239	12Aug04	Bovine	Epithelium	35.65	+	O	Oc

+ (positive); – (negative)

nucleotide sequence while the other presented a single non-synonymous mutation, resulting in an Alanine → Threonine substitution at amino acid residue 149. This residue is located in the VP1 GH loop, a site that contains the viral binding site and the most prominent neutralizing epitope [23–27]. The two isolates collected in Nangarhar differed from each other by four synonymous substitutions. There was an average 4.1% nucleotide and 4.7% amino acid difference between isolates collected on the respective borders of Afghanistan (east and west). The amino acid changes among the eastern and western serotype A viruses were distributed throughout the VP1 with two of the 10 changes located at amino acid residues 141 and 149 in the GH loop. The remaining eight changes were scattered outside of the GH loop region.

Phylogenetic reconstruction of the serotype A viruses from Hirat and Nangarhar showed that these isolates grouped together in a genetic lineage also containing viruses from Iran (Fig. 2). The closest relative, A/IRN/7/2004 (Fig. 2), was collected from the district of Zahedan in Sistan and Baluchestan province. A strong bootstrap value (87) was observed at the clade containing the isolates from Afghanistan and A/IRN/7/2004. Geographically, this area is located near the borders shared by Afghanistan, Iran and Pakistan. There was an average 3.3% nucleotide and 2.8% amino acid difference between the Afghan isolates and A/IRN/7/2004. Unfortunately, no recent (2004–2005) serotype A isolates from Pakistan were available for comparison, but serotype A viruses from 2002 and 2003 (A/IRN/2/2002 and A/IRN/7/2003) collected in north-central Iran grouped in a closely related lineage (Fig. 2).

The four serotype O viruses found in Afghanistan were collected along the major road (Ring Road) at two locations near Ghazni and Zabul (Fig. 1). Phylogenetic analysis showed that these viruses group within the previously described PanAsia O lineage of the ME-SA topotype

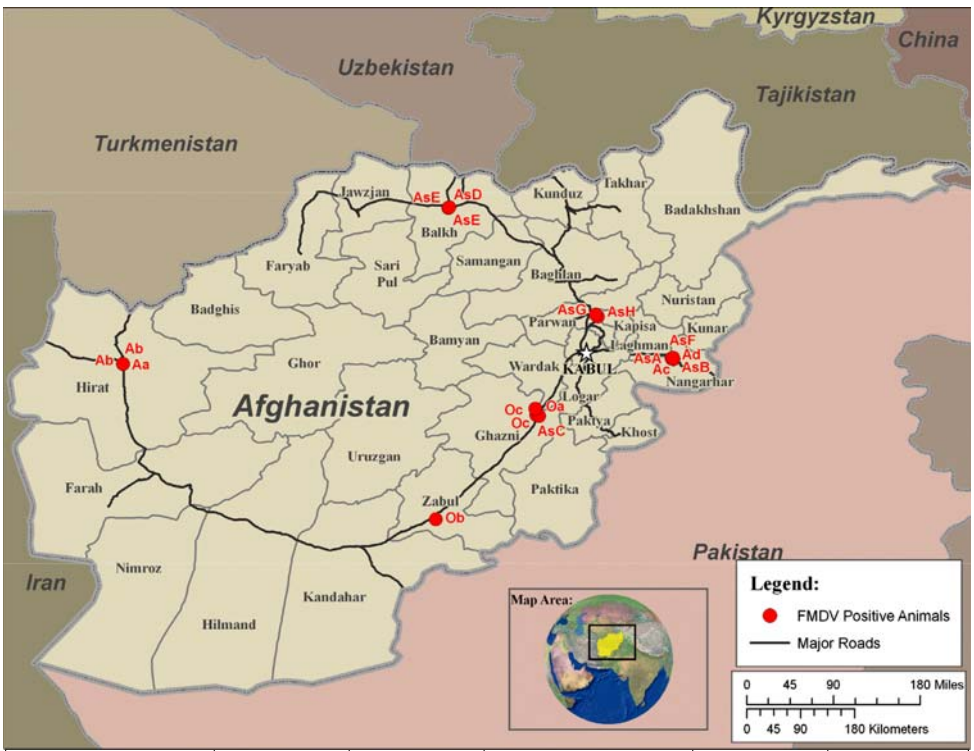

Fig. 1 Geographical distribution of VP1 sequenced Afghanistan isolates

Isolate	Province	Variant	Isolate	Province	Variant
Asia1/AFG/22/2003	Nangarhar	AsA	A/AFG/130/2004	Hirat	Aa
Asia1/AFG/24/2003	Nangarhar	AsB	A/AFG/131/2004	Hirat	Ab
Asia1/AFG/26/2003	Balkh	AsE	A/AFG/132/2004	Hirat	Ab
Asia1/AFG/27/2003	Balkh	AsE	A/AFG/160/2005	Nangarhar	Ac
Asia1/AFG/33/2003	Nangarhar	AsF	A/AFG/183/2005	Nangarhar	Ad
Asia1/AFG/40/2003	Kapisa	AsH	O/AFG/120/2004	Ghazni	Oa
Asia1/AFG/44/2003	Kapisa	AsG	O/AFG/201/2004	Zabul	Ob
Asia1/AFG/116/2004	Balkh	AsD	O/AFG/209/2004	Ghazni	Oc
Asia1/AFG/138/2004	Ghazni	AsC	O/AFG/210/2004	Ghazni	Oc

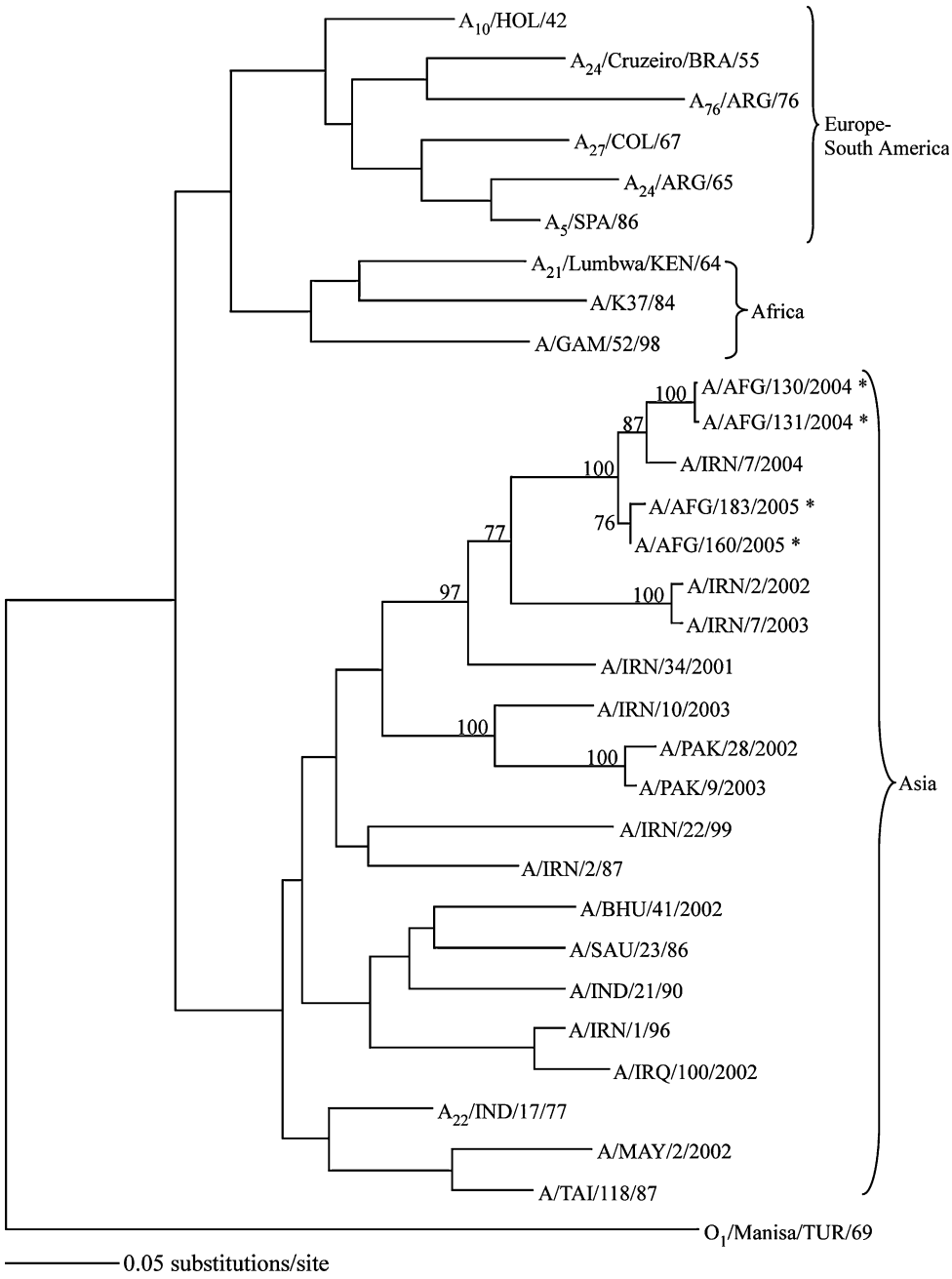

Fig. 2 Phylogenic tree (outgroup rooted on O_1/Manisa/TUR/69) showing the relationships of FMDV serotype A isolates used in this study. The relationships are based on a comparison of complete VP1 nucleotide sequences. Phylogenetic analysis was performed by maximum likelihood using PAUP*b10. Bootstrap values (2,000 replicates) are from maximum parsimony generated trees. *Isolate sequenced for this study (A/AFG/130/2004 = Aa, A/AFG/131/2004 = Ab, A/AFG/160/2005 = Ac, A/AFG/183/2005 = Ad)

(Fig. 3) [4]. Two isolates collected on the same farm in Ghazni (Fig. 1), A/AFG/209/2004 and A/AFG/210/2004, were identical in nucleotide sequence. The other two isolates, A/AFG/120/2004 and A/AFG/201/2004, collected in Ghazni and Zabul, respectively, were similar to each other with only six nucleotide changes that resulted in one amino acid change at residue 138 (Valine → Glutamic Acid) in the GH loop. A much larger divergence was observed between the A/AFG/209 and 210/2004 sequence and the other two isolates (A/AFG/120/2004 and A/AFG/201/2004). At the nucleotide level 40–41 nucleotide changes were observed which resulted in only two to three changes at the amino acid level.

O/AFG/210/2004 is most closely related to O/IRN/9/99 of the PanAsia O group [28] with 28 nucleotide changes resulting in only two amino acid changes. A bootstrap value could not be obtained for the clade this isolate fell in, but a bootstrap value of 66 was observed leading to the clade containing the "classically" defined PanAsia O serotype viruses. The two other isolates, O/AFG/120/2004 and O/AFG/201/2004, grouped with PanAsia O strains as well but were more closely related to a second genetic

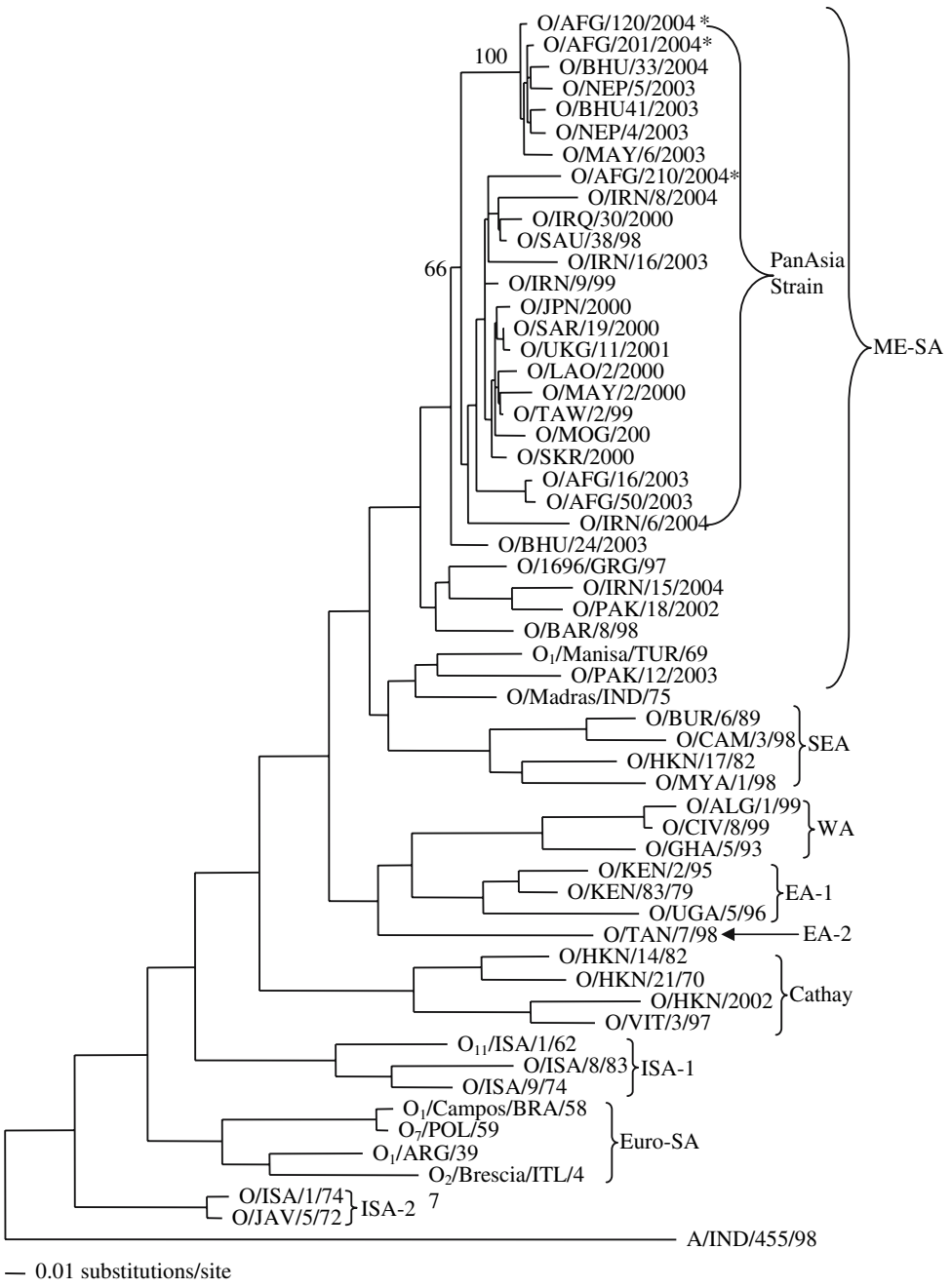

Fig. 3 Phylogenic tree (outgroup rooted on A/IND/455/98) showing the relationships of FMDV serotype O isolates used in this study. The relationships are based on a comparison of complete VP1 nucleotide sequences. Phylogenetic analysis was performed by maximum likelihood using PAUP*b10. Bootstrap values (2,000 replicates) are from maximum parsimony generated trees. *Sample sequenced at PIADC ME-SA = Middle East-South America, SEA = South-East Asia, WA = West Africa, EA-1 = East Africa-1, EA-2 = East Africa-2, ISA-1 = Indonesia-1, ISA-2 = Indonesia-2, Euro-SA = Europe-South America (O/AFG/120/2004 = Oa, O/AFG/201/2004 = Ob, O/AFG/210/2004 = Oc)

lineage that contained virus isolates collected from Nepal, Bhutan, and Malaysia during 2003–2004. A bootstrap value of 100 observed between A/AFG/120/2004, A/AFG/210/2004 and the Nepal, Bhutan, and Malaysia isolates provides strong evidence of their placement in this clade. Both of these Afghan isolates were almost identical to an isolate from Nepal, O/NEP/4/2003, with only eight synonymous changes between O/NEP/4/2003 and O/AFG/201/2004, and one amino acid change between the Nepal isolate and O/AFG/120/2004. The average nucleotide divergence observed between these two Afghan lineages was 6.3%, however, the average amino acid divergence was only 1.2%.

Asia 1 was the most frequently detected serotype in this study. Nine isolates were subdivided into eight closely related variants (termed AsA-AsH) (Fig. 4) with nucleotide and amino acid divergence ranging from 0.3–1.7% to 0.0–1.9%, respectively. Four of the eight amino acid changes observed among the eight Asia variants occurred in the antigenically relevant GH loop (Fig. 5). The Asia 1 viruses collected in the provinces of Balkh, Ghazni, Kapisa, and Nangarhar (Fig. 1) share recent ancestry and form a

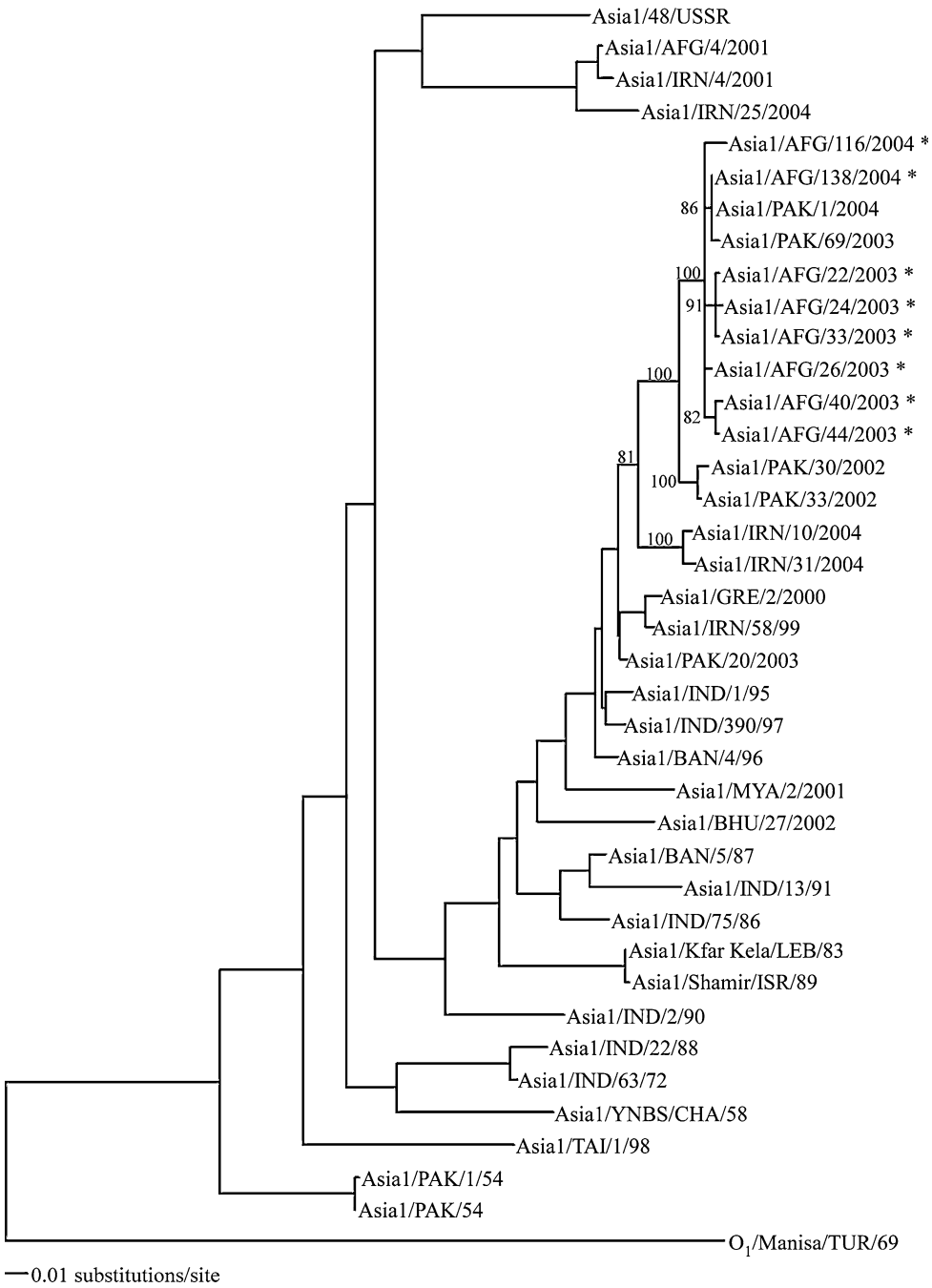

Fig. 4 Phylogenic tree (outgroup rooted on O_1/Manisa/TUR/69) showing the relationships of FMDV serotype Asia 1 isolates used in this study. The relationships are based on a comparison of complete VP1 nucleotide sequences. Phylogenetic analysis was performed by maximum likelihood using PAUP*b10. Bootstrap values (2,000 replicates) are from maximum parsimony generated trees. *Sample sequenced at PIADC (Asia1/AFG/22/2003 = AsA, Asia1/AFG/24/2003 = AsB, Asia1/AFG/26/2003 = AsE, Asia1/AFG/33/2003 = AsF, Asia1/AFG/40/2003 = AsH, Asia1/AFG/44/2003 = AsG, Asia1/AFG/116/2004 = AsD, Asia1/AFG/138/2004 = AsC)

distinct lineage with isolates circulating in Pakistan during the same time period (Fig. 4). Bootstrap values were all over 80 at the relevant nodes which provides strong evidence for the placement of the isolates within their respective clades. There were also two isolates from Iran (Asia1/IRN/10 and 31/2004) that were genetically similar (Fig. 4). The average nucleotide and amino acid divergence between the Afghan and Pakistan isolates was only 2.0 and 1.9%, respectively, and was slightly higher between Afghan and Iranian isolates (5.9 and 3.4% nucleotide and amino acid, respectively). Interestingly, the nucleotide sequence of Asia1/AFG/138/2004 (collected from the northeastern corner of Ghazni) is identical to that of Asia1/PAK/1/2004 and contains only two synonymous nucleotide changes (not leading to amino acid changes) when compared with Asia1/PAK/69/2003.

Discussion

Sequence data collected from the Afghanistan isolates in this study indicate that the three serotypes of FMDV

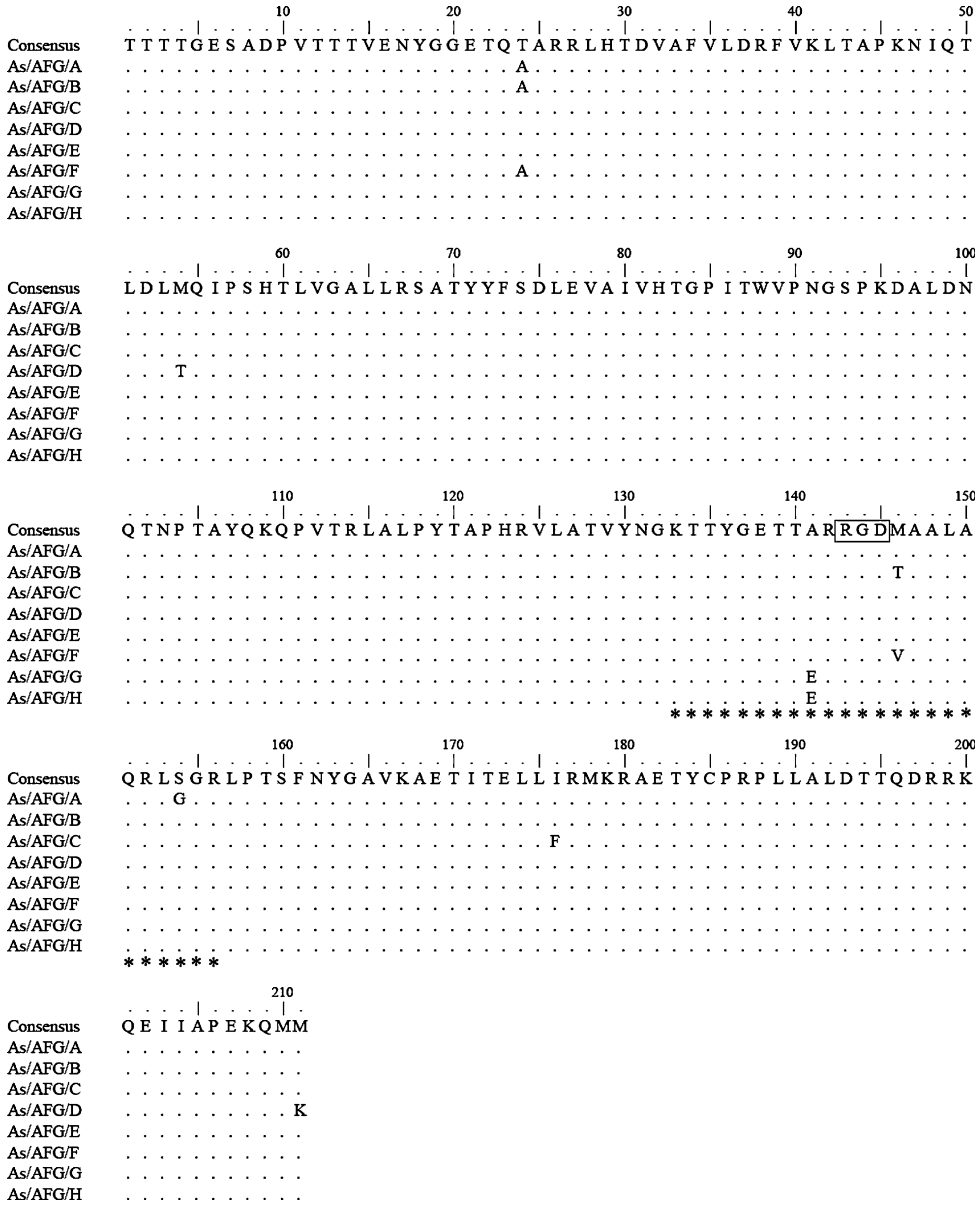

Fig. 5 Afghanistan FMDV Asia 1 amino acid analysis. Boxed Area = Arg-Gly-Asp (RGD) receptor site, *Approximate GH loop site

circulating during the period of 2003–2005 were closely related to those in surrounding countries during the same time period. These results are not unexpected given that animal movement between countries is generally unrestricted in this geographical region. For example, serotype A isolates from 2004 to 2005, although collected on the eastern and western borders of Afghanistan, were closely related and shared the same genetic lineage suggesting a common origin. Nucleotide and amino acid comparison of the VP1 region of the closest relative to the Afghan serotype A viruses (A/IRN/7/2004) showed minimal divergence (3.3 and 2.8% average, respectively). These data coupled with the fact that the serotype A viruses are known to be the most antigenically variable among the four Eurasian FMDV serotypes [4, 11, 12] provide strong evidence that the Afghan viruses collected on the western and eastern borders as well as A/IRN/7/2004 share a recent common origin. The close genetic relationship of these isolates, though collected in three distant geographic regions, indicate intra- and inter-country spread of the disease. The next closest relatives (A/IRN/2/2002 and A/IRN/7/2003) were largely divergent at both the nucleotide and amino acid level reflecting the different temporal origin of these viruses.

The presence of the PanAsia serotype O virus isolates in Afghanistan further substantiates recent observations that this strain has emerged as the dominant type O in the ME-SA region. The origin of the PanAsia O strain has been traced back to 1982 [29], and since this time, it has replaced all other serotype O strains circulating in the ME-

SA region [4, 28]. Furthermore, this viral strain has spread to distant places such as South Africa in 2000 and Europe in 2001 [30]. Type O viruses described in this report grouped into two distinct genetic lineages coexisting in Afghanistan. There are at least two possible explanations for this finding, either the PanAsia O strain is rapidly evolving, or at least two separate introductions of this strain have occurred in Afghanistan [4]. Isolates O/AFG/120/2004 and O/AFG/201/2004, while similar in both nucleotide and amino acid sequence of the VP1 region, were collected from two different provinces indicating movement of the same virus through the country. The closest relatives of these two isolates grouped with isolates from Nepal, Bhutan, and Malaysia collected during the same timeframe, illustrating the widespread movement of the PanAsia strain between countries. The second lineage, O/AFG/210/2004, which groups with the original PanAsia O strain, was closely related to O/IRN/9/99. In the absence of a more recent isolate for comparison it is difficult to determine the origin of this isolate. The low number of nucleotide changes (28) and even fewer amino acid changes (two) between the Afghan and Iranian isolate collected 5 years apart indicate genetic stability of this particular virus strain.

The Asia 1 viruses collected in Afghanistan group in a clade including isolates from Pakistan and Iran (Asia1/PAK/1/2004, Asia1/PAK/69/2003, Asia 1/PAK/30 and 33/2002, Asia1/IRN10 and 31/2004) (Fig. 4). Previously published work indicates the Asia 1 Afghanistan isolates would most likely group in the same clade as viruses responsible for causing disease in Pakistan, Iran, Tajikistan, and Hong Kong between 2002 and 2005 [10]. This group of Asia 1 viruses was genetically distinct from those isolated during the same time period in the Jiangsu province of China, the Amur province of Russia, and from those circulating in Southeast Asia and India [10]. The high similarity between Afghan isolates and their closest relatives (Pakistan and Iranian isolates) are indicative of viral spread of the same lineage. Furthermore, nucleotide and amino acid identity between isolates Asia1/AFG/138/2004 and Asia1/PAK/1/2004 provide strong evidence for a common origin of these viruses (Fig. 4).

In conclusion, this study provides a summary of the genetic diversity of recent FMDV field isolates from Afghanistan and their relationship to other FMD viruses in the ME-SA region. Assessment of the genetic variation of viruses in the field is useful for estimating the origin of outbreaks and provides valuable information applicable to control measures such as regulating animal movement and selecting appropriate vaccine strains. Additional surveillance and epidemiological studies in this region combined with proper phylogenetic analysis should aid in implementation and development of appropriate control measures.

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